

Characterization of cDNAs encoding three trypsin-like proteinases and mRNA quantitative analysis in Bt-resistant and -susceptible strains of *Ostrinia nubilalis*

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Abstract

Our previous studies suggested that *Bacillus thuringiensis* (Bt) resistance in a Dipel-resistant strain of *Ostrinia nubilalis* was primarily due to reduced trypsin-like proteinase activity. In this study, we demonstrated a 254-fold resistance to Cry1Ab protoxin but only 12-fold to trypsin-activated Cry1Ab toxin in the Dipel-resistant strain. Significantly higher resistance to Cry1Ab protoxin than to trypsin-activated Cry1Ab toxin further supports the hypothesis that reduced trypsin-like proteinase activity leading to reduced activation of the Bt protoxin is a major resistance mechanism in the Dipel-resistant strain. To understand the molecular basis of reduced proteinase activity, three cDNAs, *OnT2*, *OnT23*, and *OnT25*, encoding full-length trypsin-like proteinases, were sequenced in Bt-resistant and -susceptible *O. nubilalis* larvae. Although a number of nucleotide differences were found in sequences from the Bt-resistant and -susceptible strains, the differences were not consistent with reduced trypsin-like activity in the Bt-resistant strain. However, the mRNA levels of *OnT23* in the resistant strain were 2.7- and 3.8-fold lower than those of the susceptible strain as determined by northern blotting and real-time quantitative PCR, respectively. Thus, reduced trypsin-like activity may be attributed to reduced expression of *OnT23* in Bt-resistant *O. nubilalis*. Our study provides new insights into Bt resistance management strategies, as resistance mediated by reduced Bt protoxin activation would be ineffective if resistant insects ingest a fully activated form of Cry1Ab toxin, either in spray formulations or transgenic Bt crops.

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1. Introduction

Serine proteinases are the major midgut digestive enzymes of dietary proteins in many lepidopteran insect species (Applebaum, 1985; Terra and Ferreira, 1994).

Among the insect serine proteinases, those with trypsin and chymotrypsin specificities have been most extensively studied. They play important roles in dietary protein digestion (Terra and Ferreira 1994), as well as in the modulation of toxicity of *Bacillus thuringiensis* (Bt) toxins (Gill et al., 1992; Oppert, 1999).

The mode of action of Bt toxins in susceptible insects involves several steps, including solubilization of Bt crystals in the insect midgut, proteolytic processing of Bt protoxin by midgut proteinases, binding of activated

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toxin to midgut receptors, and insertion of the toxin molecule into the midgut epithelial cell membrane to create pores (Gill et al., 1992). Changes in the proteolytic activity of digestive enzymes can alter the toxicity of Bt toxins through effects on crystal solubilization and/or activation of protoxins, as well as degradation of activated toxin (Milne and Kaplan, 1993; Oppert et al., 1994, 1996, 1997; Martínez-Ramírez and Real, 1996; Keller et al., 1996; Forcada et al., 1996, 1999).

Our previous studies in the European corn borer, *Ostrinia nubilalis* (Hübner), demonstrated that the activity of soluble trypsin-like proteinases was significantly reduced in a strain of Dipel-resistant *O. nubilalis*, and the reduced proteinase activity was associated with reduced protoxin activation (Huang et al., 1999; Li et al., 2004a). In the present study, we further characterized the Bt resistance mechanism in the Dipel-resistant *O. nubilalis* strain by comparing the resistance levels to Cry1Ab protoxin and trypsin-activated toxin, and the expression levels of trypsin-like proteinase genes in resistant and susceptible larvae by northern blot and real-time quantitative PCR (qPCR) analyses. Results from this study further corroborated our hypothesis of proteinase-mediated resistance in this Dipel-resistant *O. nubilalis* strain.

2. Materials and methods

2.1. Insect strains

Bt-susceptible strain of *O. nubilalis* originated from egg masses collected from cornfields near St. John, Kansas, in 1995 and had been reared on artificial diet for over 47 generations, as previously described (Huang et al., 1997). The Bt-resistant strain (KS-SC) was selected from the same collections as for the susceptible strain for more than 44 generations by exposing neonates to a diet containing doses of *Bt* subsp. *kurstaki* HD-1 (Dipel, Abbott Laboratories, Chicago, IL) that resulted in 80–95% mortality. The resistance ratios to Cry1Ab protoxin and Dipel were 205 and 47, respectively (Li, 2004).

2.2. Preparation of Cry1Ab proteins

Cry1Ab protoxin was produced from a recombinant *Escherichia coli*, ECE54, which contained the *cry1Ab* gene, provided by the *Bacillus* Genetic Stock Center, Ohio State University, Columbus, Ohio. The protoxin was partially purified using the method described by Ge et al. (1990) to remove soluble proteins and other materials. Activated Cry1Ab was obtained by incubating Cry1Ab protoxin with trypsin as previously described (Li et al., 2004b). Trypsin-activated Cry1Ab

toxin was purified using anion-exchange chromatography as described by Hua et al. (2001). Cry1Ab concentration and purity were determined by densitometric analysis of the toxin band resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) and stained by Coomassie blue, using an Odyssey Infrared Imaging System (LICOR, Lincoln, NE) with bovine serum albumin (BSA) as a standard (data not shown). The molecular mass of trypsin-activated Cry1Ab toxin was approximately 58 and 132 kDa for Cry1Ab protoxin.

2.3. Bioassays with Cry1Ab proteins

Laboratory bioassays with Cry1Ab, either activated toxin or protoxin, were performed using a slightly modified diet incorporation method previously described (Huang et al., 1997). Serial dilutions of Cry1Ab protein were made in phosphate-buffered saline (PBS, pH 7.4) containing 0.1% (w/v) BSA (Denolf et al., 1993). Two milliliters of each diluted Cry1Ab protein was incorporated into 10 ml of the standard diet. The incorporated diet was dispensed into 24 cells of a 128-cell bioassay tray (Bio-Ba-128, CD International, Pitman, NJ). Two milliliters of PBS buffer containing 0.1% BSA was incorporated into 10 ml of diet as a control. Two neonates (<24-h old) were placed on the diet surface in each cell and the tray surface was sealed with perforated plastic covers (Bio-CV-16, CD International). The bioassay trays were placed in a growth chamber maintained at 25 °C, 16:8 h (light:dark) photoperiod, and 60% relative humidity. Larval mortality was recorded on the 7th day after inoculation. Five concentrations of Cry1Ab proteins were used in each bioassay and each concentration was replicated four times. Each replication included 12 neonates. The bioassays were independently performed, once with protoxin and twice with toxin. Larval mortality was corrected using the method described by Abbott (1925). LC_{50} s, 95% confidence intervals (CI), and slopes of the dose–mortality curves were determined by probit analysis (Finney, 1971) using POLO-PC statistical software (LeOra Software, 1987). Resistance ratios were calculated by dividing the LC_{50} of the resistant strain by that of the susceptible strain. Resistance was considered significant when the 95% CI for the resistance ratio did not include the value one (Robertson and Preisler, 1992).

2.4. Total RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from guts (~70 mg) dissected from 10 fifth-instar larvae of *O. nubilalis* using the NucleoSpinRNA kit (BD Biosciences, Palo Alto, CA). The first-strand cDNA (5'- and 3'-RACE-Ready cDNA) was synthesized using 4 µg of total RNA as a template

for each strain following the manufacturer's instructions of the SMARTTMRACE cDNA amplification kit (BD Biosciences). The cDNA reactions were stored at -20°C until use.

2.5. Amplification of cDNAs for trypsin-like proteinase precursors

The full-length cDNAs of three trypsin-like proteinases were amplified by a RACE-PCR strategy. The first PCR was performed to determine trypsin-like specific cDNA sequences using degenerate primers and the first-strand cDNA as a template under the PCR conditions described by Zhu et al. (1997). The forward and reverse degenerate primers were designed from two highly conserved regions of trypsin proteinases, QRIVGG and CQGDSGGP (Jones et al., 1993; Wang et al., 1993; Peterson et al., 1994; Bown et al., 1997; Zhu et al., 2000a, b; Mazumdar-Leighton and Broadway, 2001; Li et al., 2002). PCR-amplified DNA fragments (~ 600 bp) were subcloned and sequenced in both directions using an ABI PRISM 3700 DNA analyzer (AME Bioscience, Foster City, CA) at the Sequencing and Genotyping Facility, Kansas State University, Manhattan, KS. DNA sequences were compared to the GenBank database as described in the following section, and gene specific primers were designed. The full-length cDNA of each trypsin-like gene was obtained following the recommended protocol of the SMARTTM RACE cDNA amplification kit (BD Biosciences). At least five cDNA clones were sequenced in both directions for each of the three trypsin-like proteinase genes in each *O. nubilalis* strain.

2.6. Sequence analysis

The amino acid similarity search and retrieval of homologous sequences were performed using the National Center for Biotechnology Information (NCBI) Internet server (Altschul et al., 1990; Gish and States, 1993). The translation of deduced amino acid sequences was performed by using the Search Launcher software through the Baylor College of Medicine (BCM) (Smith et al., 1996; Worley et al., 1998). Alignments of DNA and deduced amino acid sequences were conducted with MULTALIN (Corpet, 1988). The signal peptide was predicted using ExPASy Proteomics Tools (Henrik et al., 1997). The sequences were deposited in the GenBank under the accession numbers AY513651 for *OnT2a*, AY513652 for *OnT2b* (*OnT2* in this paper), AY513650 for *OnT23*, and AY513649 for *OnT25*. Phylogenetic analysis was carried out with the PHYLIP software package (Felsenstein, 1993). Alignment of amino acid sequences from position 137 to 379 as demonstrated in Fig. 4 was performed with Clustal X (version 1.8) (Jeanmougin et al., 1998). Protein distances were

calculated with PRODIST using the Gonnet matrix, and a phylogenetic tree was constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987). Bootstrap values were calculated with SEQBOOT program using 1000 replications. The tree was drawn by the program TreeView (Page, 1996).

2.7. Northern blot analysis

Expression levels of the RNA encoding trypsin-like proteinases *OnT2*, *OnT23*, and *OnT25* in fifth-instar larvae of Dipel-resistant and -susceptible *O. nubilalis* were compared by northern blot analysis using a NorthernMaxTM kit (Ambion, Austin, TX) according to manufacturer's instructions. Total RNA from each strain (25.0 $\mu\text{g}/\text{lane}$) was separated in a 1.2% agarose gel supplemented with denaturing buffer. Radioactive cDNA probes were prepared by a nick translation (Invitrogen) using α -³²P-dCTP. Full-length cDNAs were used as probes for *OnT23* and *OnT25*, whereas a 507-bp cDNA fragment, amplified by PCR with *OnT2*-specific primers *OnT2F* (5'-CGC AAA GCC ACG ATT TTC AAG TGT) and *OnT2R* (5'-TTA TAG CCG CAG CTG CAG GTG GGG), homologous to neither *OnT23* nor *OnT25* cDNAs, used as a probe for *OnT2*. After hybridization and washing, the membrane was exposed with an intensifying screen to Kodak X-Omat AR film at -80°C for 12 h for *OnT2*, and 3 h for *OnT23* and *OnT25*. To check the normalized loading, membranes were stripped with a boiling solution of 5% SDS, and a radiolabeled ribosomal protein S3 (RPS3) cDNA (~ 700 bp) from tobacco hornworm, *Manduca sexta* (Jiang et al., 1996, provided by Dr. M. Kanost, Kansas State University) was incubated with the stripped membrane. The membranes were washed and exposed to X-ray film for 12 h at -80°C . Northern blot analysis was repeated three times with total RNA extracted from three groups of 10 fifth-instar larvae from different generations of each strain. All X-ray films were scanned, and their intensities and areas were measured using an infrared image system (Li-Cor, Lincoln, NE). The band intensities for *OnT2*, *OnT23*, and *OnT25* in the resistant and susceptible strains were normalized using the RPS3 internal reference. Normalized relative intensity data were compared by the Student *t*-test (SAS Institute, 1990).

2.8. Real-time quantitative PCR

Real-time qPCR (Heid et al., 1996; Wang and Brown, 1999) was performed to further compare expression levels of *OnT23* in the two strains. Plasmids containing full-length *OnT23* cDNA were used as a template to establish standard curves and determine the copy numbers of *OnT23* cDNA in samples. A forward primer, RTqF (5'-AAC GTG GCC AAT GAA TAC

AAC), and a reverse primer, RTqR (5'-ATT GTC ACG TGC CTC AGA AC), were designed to amplify a 227-bp fragment using the OligoPerfectTM primer designer (www.Invitrogen.com).

Similarly, RPS3 was also included to normalize the cycle threshold (C_t) values for *OnT23* amplification between the two strains. The RPS3 used in real-time qPCR was isolated from the susceptible strain of *O. nubilalis* using ProSTARTM Ultra HF RT-PCR System (Stratagene, La Jolla, CA). From highly conserved regions of nucleotide sequences of RPS3 found in *M. sexta* (P48153, Jiang et al., 1996) and the fall armyworm, *Spodoptera frugiperda* (AF429976, Landais et al., unpublished), a pair of degenerate primers (forward: 5'-AGC TKG CTG AGG ATG GCT ACT; reverse: 5'-CYA CGA ACT TCA TKG ACT TGG) were used to amplify a 379-bp cDNA fragment from the susceptible strain of *O. nubilalis*. PCR was performed using specific primers to obtain RPS3 cDNA from *O. nubilalis*. This RPS3 sequence was deposited in the GenBank under the accession number AY513653. A pair of specific forward and reverse primers was then designed to amplify a 209-bp fragment in real-time qPCR.

For preparation of templates for qPCR, total RNA was extracted and purified from three groups of six fifth-instar larvae of different generations of each strain of *O. nubilalis*, as previously described. Ten micrograms of total RNA from each sample was used to synthesize the first-strand cDNA using an oligo(dT) primer (18 mer) in a 20- μ l reaction following the ProSTARTM Ultra HF RT-PCR System (Stratagene). The first-strand cDNA reaction was stored in 5- μ l aliquots at -20°C until use.

Real-time qPCR was carried out with Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) (Wittwer et al., 1997) in a 25- μ l reaction using a Cepheid Smart Cycler (Cepheid, Sunnyvale, CA). Recombinant pCR 2.1 TOPO plasmid containing full-length *OnT23* was used as a template to establish a standard curve for each PCR. After preliminary experiments to optimize reaction factors, a range of DNA concentrations, from 1.0 ng to 10.0 fg (equivalent to 1.97×10^8 to 1.97×10^3 copies

per reaction mixture) was utilized to generate a set of standard curves for each PCR. The concentration of each primer was 100 nM for amplification of either *OnT23* or RPS3. One microliter of a 20- μ l first-strand cDNA reaction was used as a template for all reactions. Modified touchdown PCR programs (Don et al., 1991) were used for both RPS3 and *OnT23*. The programs consisted of $50^{\circ}\text{C}/120\text{ s}$ and $95^{\circ}\text{C}/120\text{ s}$ for initiation; five cycles of $95^{\circ}\text{C}/15\text{ s}$, $62^{\circ}\text{C}/10\text{ s}$ for RPS3 and $60^{\circ}\text{C}/10\text{ s}$ for *OnT23*, and $72^{\circ}\text{C}/15\text{ s}$; five cycles of $95^{\circ}\text{C}/15\text{ s}$, $60.5^{\circ}\text{C}/10\text{ s}$ for RPS3 and $58.5^{\circ}\text{C}/10\text{ s}$ for *OnT23*, and $72^{\circ}\text{C}/15\text{ s}$; 40 cycles of $95^{\circ}\text{C}/15\text{ s}$, $59^{\circ}\text{C}/10\text{ s}$ for RPS3 and $57^{\circ}\text{C}/10\text{ s}$ for *OnT23*, and $72^{\circ}\text{C}/15\text{ s}$; and $72^{\circ}\text{C}/120\text{ s}$ for a final extension, a total of 50 cycles. Melting curves were obtained by increasing the temperature from 60 to 95°C ($0.2^{\circ}\text{C}/\text{s}$) to denature the double-stranded DNA.

The specificity of each reaction was evaluated based on the melting temperatures of the PCR products (Ririe et al., 1997). C_t values from specific standard reactions were used to establish a regression curve with the initial DNA copy number in log scale. Regression curves were used to estimate the initial cDNA copy numbers in each sample reaction. Normalized C_t values of *OnT23* for the resistant and susceptible strains were obtained by adding or subtracting the deviation proportion of the C_t values of RPS3 in the two strains. The experiment was repeated three times with three pairs of the first-strand-cDNA samples from different generations of resistant and susceptible larvae. The average of copy numbers of *OnT23* mRNA in the resistant and susceptible was analyzed with the Student *t*-test (SAS Institute, 1990).

3. Results

3.1. Susceptibility to *Cry1Ab* protoxin and trypsin-activated toxin

The results of bioassays with *O. nubilalis* larvae using different forms of *Cry1Ab* indicated that the susceptible strain was more sensitive to *Cry1Ab* protoxin and toxin

Table 1
Susceptibility of Dipel-resistant and -susceptible *O. nubilalis* larvae to *Cry1Ab* protoxin and toxin

Insect strain	Slope \pm SE	LC ₅₀ (95% CI) ^a ($\mu\text{g}/\text{ml}$)	χ^2	df ^b	Resistance ratio (95% CI) ^c
Protoxin					
Susceptible	1.33 ± 0.15	4.67 (3.15–6.94)	23.0	22	
Resistant	1.09 ± 0.15	1190 (815–1880)	15.3	22	254 (151–427)
Toxin					
Susceptible	1.40 ± 0.13	0.58 (0.46–0.80)	38.7	46	
Resistant	1.27 ± 0.12	6.92 (5.29–9.31)	38.7	46	11.9 (8.41–16.7)

^aLC₅₀ was the concentration of *Cry1Ab* that killed 50% of *O. nubilalis* larvae, with 95% confidence intervals.

^bAll critical values of χ^2 at each df value, $P = 0.05$ were greater than the respective χ^2 value that was calculated by POLO program, indicating that the observed regression model significantly fit expected probit models.

^cResistance ratio = LC₅₀ for resistant strain/LC₅₀ for susceptible strain, with 95% confidence intervals.

than the resistant strain (Table 1). The LC_{50} values of Cry1Ab protoxin were 4.67 $\mu\text{g}/\text{ml}$ for the susceptible strain and 1190 $\mu\text{g}/\text{ml}$ for the resistant strain with a resistance ratio of 254. For Cry1Ab toxin, the LC_{50} values were 0.58 $\mu\text{g}/\text{ml}$ for the susceptible strain and

6.92 $\mu\text{g}/\text{ml}$ for the resistant strain, a resistance ratio of 11.9. These data reflected more than a 95% loss of resistance when trypsin-activated Cry1Ab was fed to resistant insects as compared to the protoxin bioassay results.

(A)	-144	AAGCAGTGGTATCAACGCAGAGTACGCGGGATTCGCTATCAGTTGTTTATGTGTTA	-88
		G <i>OnT2F</i> →	
		ATGAACGCCAGACAATTAATACAGCGGATCGCAAAGCCACGATTTTCAAGTGT	3
	-87		M -18
		TGGATCTCACTAGCCTTTGCGCTAGTATTGACGATCGGGACATCATCAGCTCAAGACGCAAACTGCGACTTTGTTTCAATGTGCAAGCA	93
	-17	W I S L A F A L V L T I G T S S A Q D A N C D F V S N V Q A	13
		... -3 -2 -1 1 2 3 ...	
		C G A T T C	
		GGTCAGACCTACTATGTGTACAGTCCAACTACCCCCAGAACTACAGGCCAGGAGTGCAGTGCCGCTGGGTCCGCATCTGTCCCTCCGGG	183
		G Q T Y Y V Y S P N Y P Q N Y R P G V Q C R W V G I C P S G	43
		S	
		C	
		TACAACTGCCGGCTAGACTGCAATGATATCTCTTGGCCACAGACAAGTGGCTGCTCTCTCGACCGTCTGCTGATATCCAAGTCCGGTGAC	273
		Y N C R L D C N D I S L P Q T S G C S L D R L L I S K S G D	73
		T	
		CCTCAGTTGACGTGACGACAGTACTACTGCGGCACAGGGACTGTACCGCCGTGTCTACTGGCCAGAGGATCAGCGTGGGGTTGATAACG	363
		P Q L T S A D Y Y C G T G T V T A V S T G Q R I S V G L I T	103
		← <i>OnT2R</i>	
		TCGACGCAAACTCTGGAGGAAGATTCATGTGCCAGCTGACCGCGCAGGCCGCCACGACAAACCCACCTGCAGCTGCGGCTATAAGAAA	453
		S T Q S P G G R F M C Q L T A Q A A T T N P T C S C G Y K K	133
		G	
		ACAAACCGTATAGTCGGAGGGCAACAGACAGGCGTGAACGAATTCCTCAATGATGGCTGGTCTGGCCCATAAAGATATAGCGCAGATCAAG	543
		T N R I V G G Q Q T G V N E F P M M A G L A H K D I A Q I K	163
		↑	
		TGCGGCGCCGTATCATCAGCAAGCGATACGTGATGACCGCGCCCACTGTCTGACAGGCGAGAGCTTGAGCAACCTGGCCATTATCGTG	633
		C G A V I I S K R Y V M T A A H C L T G Q S L S N L A I I V	193
		GGGAGCATGACGTACCGTTGGAGATTCTCCAGCAACCAAGGATTTTCAGGTATCTCTGCAATAATTCATCCCAATTACACGCGCTCG	723
		G E H D V T V G D S P A T Q G F Q V I S A I I H P N Y T P S	223
		AACTACGATTATGACATAGCGATCTGAAAACGAACGCGGACATAAATTTTCAGTGACCGCGTGGCGCCCGTCTGCTTACCTTTCAAAATTC	813
		N Y D Y D I A I L K T N A D I T F S D R V G P V C L P F K F	253
		T	
		GTCAACACCGACTTCACCGGATCCAACTTACTATTTTGGGATGGGGGACTCAATTCCCTGGCGGACCGACGTCTAACTACCTCCAGAAG	903
		V N T D F T G S K L T I L G W G T Q F P G G P T S N Y L Q K	283
		L	
		G	
		GTAGACGTGGATGTGATCAGCCAGACCTCGTGCAGGAATGTGGTGGCCACTGTGACGGCACGACAGATCTGTACTTACACCCAGGGAAA	993
		V D V D V I S Q T S C R N V V P T L T A R Q I C T Y T P G K	313
		S	
		GACGCTTGCCAGGACGACTCCGGCGGCCCATTTGCTCTACACGAGTCCAGCAACGGCCTGCTATACAGCATCGGCATCGTCAGCAACGGG	1083
		D A C Q D D S G G P L L Y T D S S N G L L Y S I G I V S N G	343
		CA G AC	
		CGTTTCTGCGCCGGCGCCCAACAGCCGGGCGTCAACACGCGGGTGCCGGCGCTGCTATCTGGATCCAAACAAACACTCCCCAGCCAGC	1173
		R F C A G A N Q P G V N T R V P A L L S W I Q T N T P D A S	373
		T N	
		TACTGCTATAAATGATTTCTGAACCCACCGTGTTTTATTCAACTTACAAATTACAACCAATAAACCTTAAGCTCGCGCTAACTGTGCGGCA	1263
		Y C Y K	377
		GTACCTAGTCAGTGTACTGGCATCTTGCAAAACAATAAACCCAAAAA	1333

Fig. 1. Nucleotide and deduced amino acid sequences of trypsin-like cDNA *OnT2* (A), *OnT23* (B), and *OnT25* (C) from Bt-resistant and -susceptible strains of *O. nubilalis*. ATG = start codon; TGA or TAA = stop codon; AATAAA or AATATA = polyadenylation signal; ↑ = predicted activation peptide cleavage site; IVGG = N-terminal residues of the mature proteinases. The predicted signal peptide is underlined. Bold-faced nucleotides and bold-faced and italicized amino acids indicate variations that were observed in cDNAs from different individuals or different strains. In panel A, *OnT2F* and *OnT2R* were the primers used to obtain a probe for northern blot analysis. In panel B, RTqF and RTqR were the primers used in the real-time qPCR analysis. Hyphens represent deletion of the nucleotides at the corresponding positions. In panel C, 20 amino acids underlined with a short line were compared with the N-terminal sequence of a purified trypsin proteinase by Bernardi et al. (1996), and the difference in amino acid indicated by a square.

(B)	-31	CCGCGGGGAGTAAAGACAAGGCTGTCAAAT	-1
ATGGTGCCTCTCGTGTCTGCTGACTTTGGCGTTATTTCAGGATGCTGTACGCAGCGCCAGAAATCGTTGGGGGGCAGGAGACCACCATC	90		
M V R L V L L T L A L F A G C C Y A A P R I V G G Q E T T I	12		
-18	...	-3 -2 -1 1 2 3 ↑ ...	
AACGAGTATCCATCCATCGTCCAGGTGGAGTTCCTGGGAATCTTCTCACAGGCTGGTCGCAGTGTGTGCTAACATCCTCAGCTCC	180		
N E Y P S I V Q V E F L G I F S Q A W S Q S C A A N I L S S	42		
AGATACGTGCTGTCTGCTGCCCCATTGCTTTGCTGGCATCTTCTACTCACCCCTCCCTACGCCGATTCGAGCTGGCACCACCTTCCGCAAC	270		
R Y V L S A A H C F A G I F Y S P S L R R I R A G T T F R N	72		
RTqF →			
TCCGGTGGGTTTACCAGAAACGTGGCCAATGAATACAACCAACCCACCTATGGACTGTTGGGGGGCCGACGGAGACATCACCGTTGTGAGG	360		
S G G F T R N V A N E Y N H P T Y G L L G A D G D I T V V R	102		
CTGGCGGAGCCTTTGGAATACAACCCAGTCCAGGAGGGTACATCGCCAACCCGAAACACAGTGATCCCTGATAACAGCCTGTGATC	450		
L A E P L E Y N P V V Q A G Y I A N P N T V I P D N Q P V I	132		
← RTqR			
CACGCTGGGTGGGACACACGCAGTTCGGAGGACACCCATCCGACGTTCTGAGGCACGTGACAACTCTTACCATCAACCACGCCATCTGC	540		
H A G W G H T Q F G G H P S D V L R H V T I F T I N H A I C	162		
CGCGACCGCTACGCAACTCTTGGCTGGCCCGTCACTGAGAACATGATCTGTGCTGGTCTTCTTGACGTGGGAGGCCGAGACGCCTGCCAG	630		
R D R Y A T L G W P V T E N M I C A G L L D V G G R D A C Q	192		
GGAGACTCTGGTGGACCTCTGTACCATGGCTCCACCCTCGTCCGTTGTTCTCTTGGGGCGAAGGCTGCGCGAACGCCACTTTCCTTGGGA	720		
G D S G G P L Y H G S T L V G V V S W G E G C A N A T F P G	222		
GTCAGCACTGCGGTGCGCGCCCTACACTTCTTGGATTTGTTTCTGTTGCTGTTTAAGGAAATACCTCACAAGTTATCTATTGGATTAAATTC	810		
V S T A V A P Y T S W I V S V A V	239		
TTCAACTTCAAGCTCCAGTGTTCGGTAAATTTGAATCAACAATAACTAAGCAAATATGTTTCATACCATTCGTTGTGAATAAAAGCCAAAT	900		

AAATGAATAAATGAATTAAAGCATTGTGTGACATGTTTGAATGACCGGTTAATAAAAAAAAAAAAAAAAAAAAAA	975		

Fig. 1. (Continued)

3.2. Trypsin-like proteinase cDNAs

Three cDNA fragments were obtained that encoded polypeptides with amino acid sequences similar to trypsin-like proteinase precursors found in other organisms. Sequence analysis of the three cDNA fragments indicated identities ranging from 43% to 54%, suggesting that these cDNAs were derived from three different trypsin-like genes. Full-length sequences of the three cDNAs were designated as *OnT2*, *OnT23*, and *OnT25*, and sequences of each were obtained from susceptible and resistant *O. nubilalis* larvae (Fig. 1).

OnT2 cDNA consisted of 1477 nucleotides with an open reading frame (ORF) of 1185 nucleotides that encoded 395 amino acid residues. The cDNA sequences from both strains included the ATG start codon and the stop codon TGA at position 1186–1188, and two polyadenylation signals, AATAAA, at positions 1231–1236 and 1296–1301 (Fig. 1A). *OnT23* cDNA consisted of 1006 nucleotides with an ORF of 771 nucleotides that encoded 257 amino acid residues. The sequences of *OnT23* from both strains had the ATG start codon and the stop codon TAA at position 772–774, and three polyadenylation signals, AATAAA,

at 887–892, 898–903, and 906–911 (Fig. 1B). *OnT25* cDNA consisted of 874 nucleotides with an ORF of 768 nucleotides that encoded 256 amino acid residues. The start codon, stop codon, and polyadenylation signal are indicated in Fig. 1C. The identities of nucleotide sequences for *OnT2* and *OnT23*, *OnT2* and *OnT25*, and *OnT23* and *OnT25* were 44%, 44%, and 51%, respectively.

3.3. Comparison of mRNA expression of the three trypsin-like proteinases

Northern blot analyses demonstrated transcripts of 1.5 kb for *OnT2* and 0.9 kb for both *OnT23* and *OnT25* (Fig. 2A). These transcripts corresponded to the predicted sizes of mRNAs based on the cDNA sequences. Among the three transcripts in both strains, the highest expression of mRNA was *OnT25*, followed by *OnT23* and *OnT2*. The level of *OnT23* mRNA in the resistant strain was 2.7-fold lower than that in the susceptible strain (Fig. 2B) ($P < 0.01$). However, there were no significant differences in mRNA levels of *OnT2* and *OnT25* ($P > 0.05$).

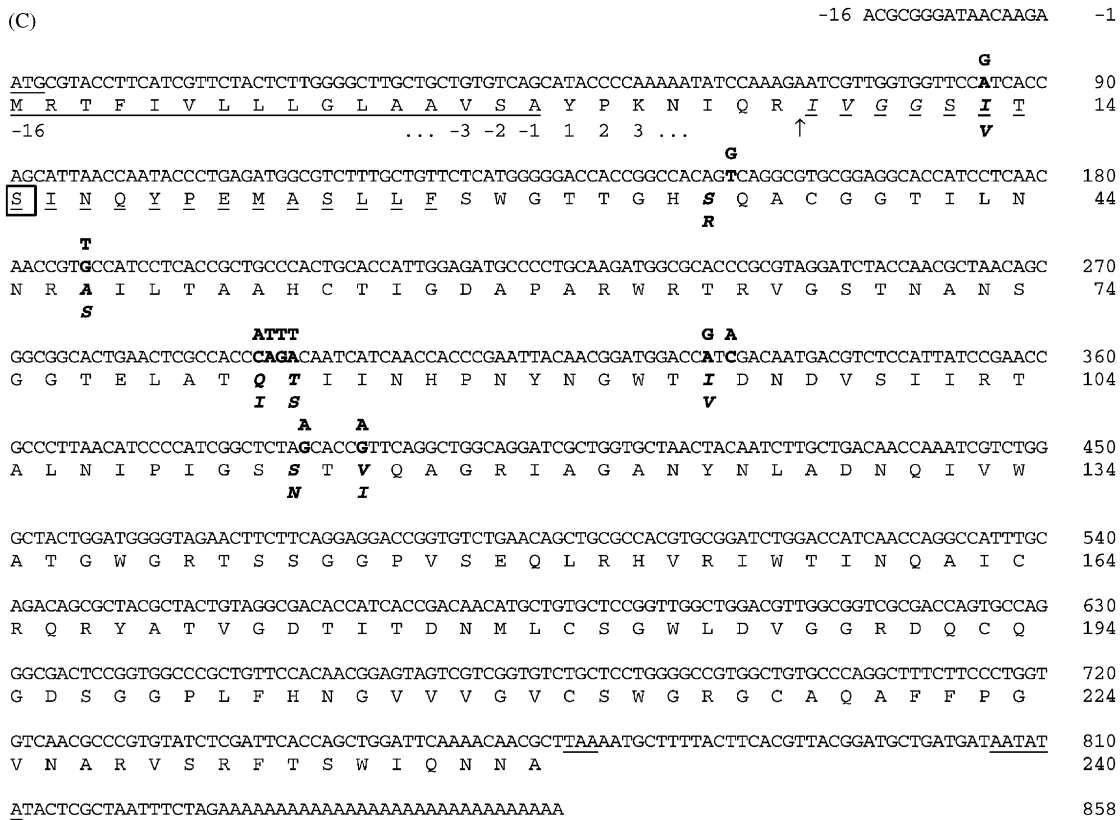


Fig. 1. (Continued)

Real-time qPCR analyses (Fig. 3) confirmed the northern blot analysis results for *OnT23* mRNA expression. The amplification curves of *OnT23* DNA fragments from the plasmid (pCR2.1-TOPO) DNA template at the range of starting copy numbers from 1.97×10^8 to 1.97×10^3 were sequentially distinctive (data not shown). Except for the control reaction with no plasmid DNA, all amplifications were considered specific by melting curve analysis, and C_t values from these amplification curves and the starting DNA copy numbers in log scale were used to generate a log-linear regression plot for each replication (Fig. 3A). All regressions of log starting copy number and C_t values were significantly linear ($R^2 > 0.98$, $P < 0.01$). The three log-linear regression plots for the three replications were similar, with intercepts of 45.1, 46.5, and 47.8 and slopes of 3.7, 4.1, and 3.9, respectively.

The copy numbers of the first-strand cDNA of *OnT23* were estimated according to the normalized C_t values and corresponding linear regression equations for resistant and susceptible strains. The copy number of the first-strand cDNA of *OnT23* was 3.8 ± 1.6 -fold lower in the resistant strain than in the susceptible strain (Fig. 3B), a difference that was statistically significant ($P < 0.05$).

3.4. Deduced protein sequences

The predicted trypsin-like proenzymes from *O. nubilalis* strains have an activation peptide with arginine in the activation cleave site (Fig. 1). The deduced amino acid sequence from *OnT2* contained 395 residues that consisted of a putative 18-residue signal peptide and a 377-residue trypsin-like proenzyme (Fig. 1A). The mature enzyme contained 241 amino acids, including 17 negatively charged residues (aspartic acid and glutamic acid) and 14 positively charged residues (arginine and lysine). The predicted molecular mass and theoretical pI value were 26 kDa and 6.05, respectively. The deduced amino acid sequence from *OnT23* consisted of 257 amino acid residues, including a putative 18-residue signal peptide and a 239-residue trypsin-like proenzyme (Fig. 1B). The mature enzyme contained 236 amino acids, including 15 negatively and 11 positively charged residues. The molecular mass and theoretical pI value were 25 kDa and 5.95, respectively. The deduced amino acid sequence encoded by *OnT25* comprised a putative 16-residue signal sequence and a 240-residue trypsin-like precursor (Fig. 1C). The mature enzyme had 233 amino acids with 12 negatively and 15 positively charged residues, with a predicted molecular mass and theoretical pI value of 25 kDa and 8.27, respectively.

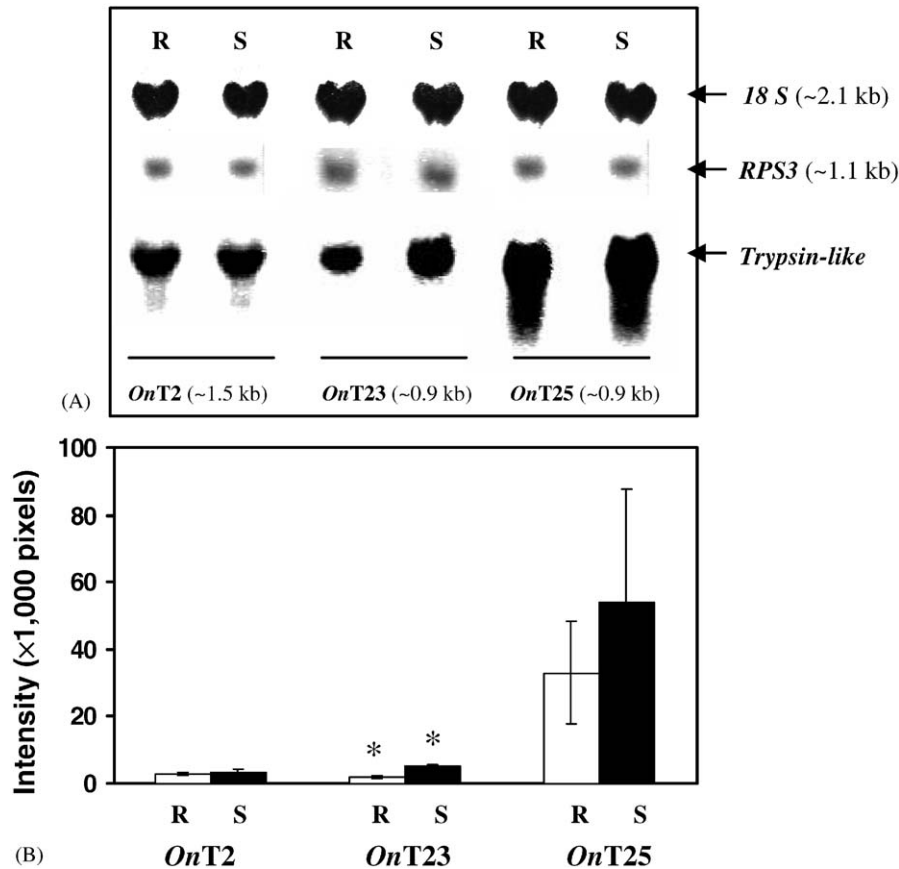


Fig. 2. Northern blots (A) of *OnT2*, *OnT23*, and *OnT25* mRNA from Bt-resistant (R) and -susceptible (S) strains of *O. nubilalis* and densitometric comparison (B). Each lane was loaded with 25.0 µg total RNA. RPS3 and 18S RNAs were used as an internal loading reference. The error bars represent standard errors. The stars (*) indicate the significant difference in the mRNA blot intensity between the two strains as determined by Student *t*-test ($P = 0.0037$). In Panel A, the relative location of each blot does not represent the molecular size of RNA. The hybridized membrane exposed to X-ray film for 12 h for *OnT2* and 3 h for both *OnT23* and *OnT25*.

The multiple sequence alignment of the trypsin-like precursors of *O. nubilalis* with three homologous trypsin-like precursors from other insect species indicated that all three amino acid sequences (*OnT2*, *OnT23*, and *OnT25*) contain the conserved residues of typical trypsin proteinases (Fig. 4). These residues included a highly conserved *N*-terminal sequence (IVGG), three conserved active site residues, His¹⁸³, Asp²³⁴, and Ser³³⁴, which form the catalytic triad in serine proteinases (Kraut, 1977; Wang et al., 1993; Peterson et al., 1994; Zhu et al., 2000b), and six cysteine residues. The residues Asp³²⁸, Gly³⁵⁷, and Gly³⁶⁷, which define the substrate-binding pocket, are conserved in *O. nubilalis* trypsin-like proteinases (Hedstron et al., 1992; Wang et al., 1993; Zhu et al., 2000b).

Phylogenetic analysis demonstrated that *OnT23* clusters with the Bt resistance-associated trypsin-like proteinase in *P. interpunctella*. In addition, *OnT23* and *OnT25* are more closely related and are in the same cluster of trypsin genes of Lepidoptera, while *OnT2* is in the cluster of dipteran and hemipteran insects (Fig. 5).

3.5. Comparison of *O. nubilalis* trypsin cDNAs and deduced protein sequences in resistant and susceptible strains

The cDNA sequences of *OnT2*, *OnT23*, and *OnT25* from resistant and susceptible strains differed within the strains as well as between the two strains (Fig. 1). There were 16 nucleotide variations in five *OnT2* cDNA sequences from each strain. Fifteen variations occurred within the ORF, six of which resulted in changes in the predicted amino acid residue, and four amino acid variations in the predicted mature trypsin-like protein. In addition, there was an *EcoR* I site, (492)GAATTC(499), due to the substitution of G→A⁴⁹⁵ in *OnT2b*, that was not found in *OnT2a*. Despite these differences, the *pI* value and number of negatively or positively charged residues were not changed in the predicted mature proteins. For *OnT23*, there were 12 nucleotide differences, including one deletion or an insertion of a short nucleotide sequence, (906)ATAAATGA(915). Nine out of the 12 variations

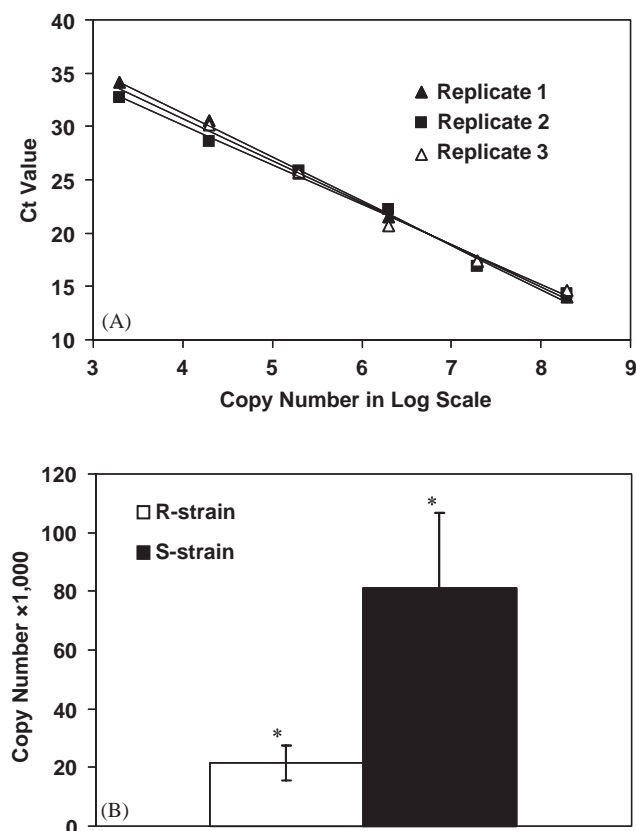


Fig. 3. Real-time quantitative PCR analysis of the mRNA expression of trypsin-like *OnT23* proteinase in the Bt-resistant (R) and -susceptible (S) strains of *O. nubilalis*. (A) Regression plots of cycle threshold (C_t) values against the starting copy numbers (in log scale) of *OnT23* plasmid DNA. Regression analysis of three replicates resulted in the following equations: $y = 45.1 - 3.7x$ ($R^2 = 0.99$, $P < 0.01$, ■); $y = 47.8 - 4.1x$ ($R^2 > 0.99$, $P < 0.01$, ▲); and $y = 46.5 - 3.9x$ ($R^2 = 0.99$, $P < 0.01$, △), where y refers to the C_t value and x to the copy number of DNA in log scale. (B) Mean of the corrected copy number of *OnT23* from first-strand cDNA shows a significant difference ($P = 0.02$) between resistant and susceptible strains of *O. nubilalis* as indicated by stars (*). Error bars represent standard errors.

occurred within the ORF, and only A→T⁴¹⁸ and C→A⁵⁶⁹ resulted in different amino acid residues, Asn→Tyr¹²² and Pro→His¹⁷². For *OnT25*, there were 11 differences in the ORF of the cDNA sequences, and eight resulted in amino acid differences. A change of Arg→Ser³⁵ resulted in an increase in the *pI* of the mature protein from 8.27 to 8.58. However, these variations were found not only between the resistant and susceptible strains but also within each strain.

4. Discussion

The most commonly documented Bt resistance mechanism is the alteration of midgut receptor binding for Bt toxins (Ferré et al., 1991; Lee et al., 1995; Gahan et al., 2001; González-Cabrera et al., 2003;

Morin et al., 2003). However, several studies have associated Bt resistance with reduced activity of digestive enzymes involved in the solubilization and activation of Bt protoxins (Oppert et al., 1994, 1996, 1997; Forcada et al., 1996, 1999; Herrero et al., 2001). In *O. nubilalis*, extensive comparisons in the number of Bt toxin binding proteins, binding affinity, binding site concentration, and overall binding capacity of the brush border membrane revealed no significant differences between the Dipel-resistant and -susceptible larvae (Li et al., 2004b). Therefore, we concluded that resistance to Cry1Ab and Cry1Ac in this resistant strain was not associated with a loss of receptor binding of Cry1Ab or Cry1Ac protein.

However, we found that trypsin-like proteinase activity was reduced by more than 50% in the Dipel-resistant strain compared to the susceptible strain, resulting in reduced Cry1Ab protoxin activation in vitro (Li et al., 2004a). In this study, we demonstrated that Dipel-resistant *O. nubilalis* larvae were over 95% more susceptible to trypsin-activated Cry1Ab toxin than to full-length Cry1Ab protoxin. All of these data support the hypothesis that reduced trypsin-like proteinase activity leading to the reduced activation of the Bt protoxin is the primary resistance mechanism in the Dipel-resistant *O. nubilalis* strain. Although trypsin is a major enzyme for dietary protein digestion in lepidopteran insect midguts, the reduction of trypsin-like proteinase activity in the Dipel-resistant *O. nubilalis* larvae did not result in a significant effect on the growth and development of the resistant insects on non-toxic diet (Huang et al., 2005).

Similar to the reduced trypsin-like proteinase activity, this study also demonstrated a significantly reduced level of *OnT23* mRNA in Dipel-resistant *O. nubilalis* compared to the susceptible strain. Therefore, we proposed that the reduced trypsin-like activity may be a result of reduced mRNA expression of *OnT23*. Similar findings have been documented in other insect species. For example, the expression of a trypsin-like gene (*PiT2c*) was reduced by 2.8-fold in a Bt-resistant strain (HD198r) of *P. interpunctella* when compared to that of a corresponding susceptible strain (Zhu et al., 2000a). Reduced trypsin-like activity contributed as much as 90% of the resistance to Cry1Ab protoxin in that strain (Herrero et al., 2001). *OnT23* in resistant *O. nubilalis* was most similar to *PiT2c* in resistant *P. interpunctella*, among all available insect trypsin sequences in the GenBank database, and both *PiT2c* and *OnT23* are associated with resistance to Cry1Ab protoxin. Further studies are needed to examine the physiological significance of these closely related trypsin-like genes and resistance to Bt toxins.

To characterize the molecular basis of reduced trypsin-like activity, we compared sequences of three

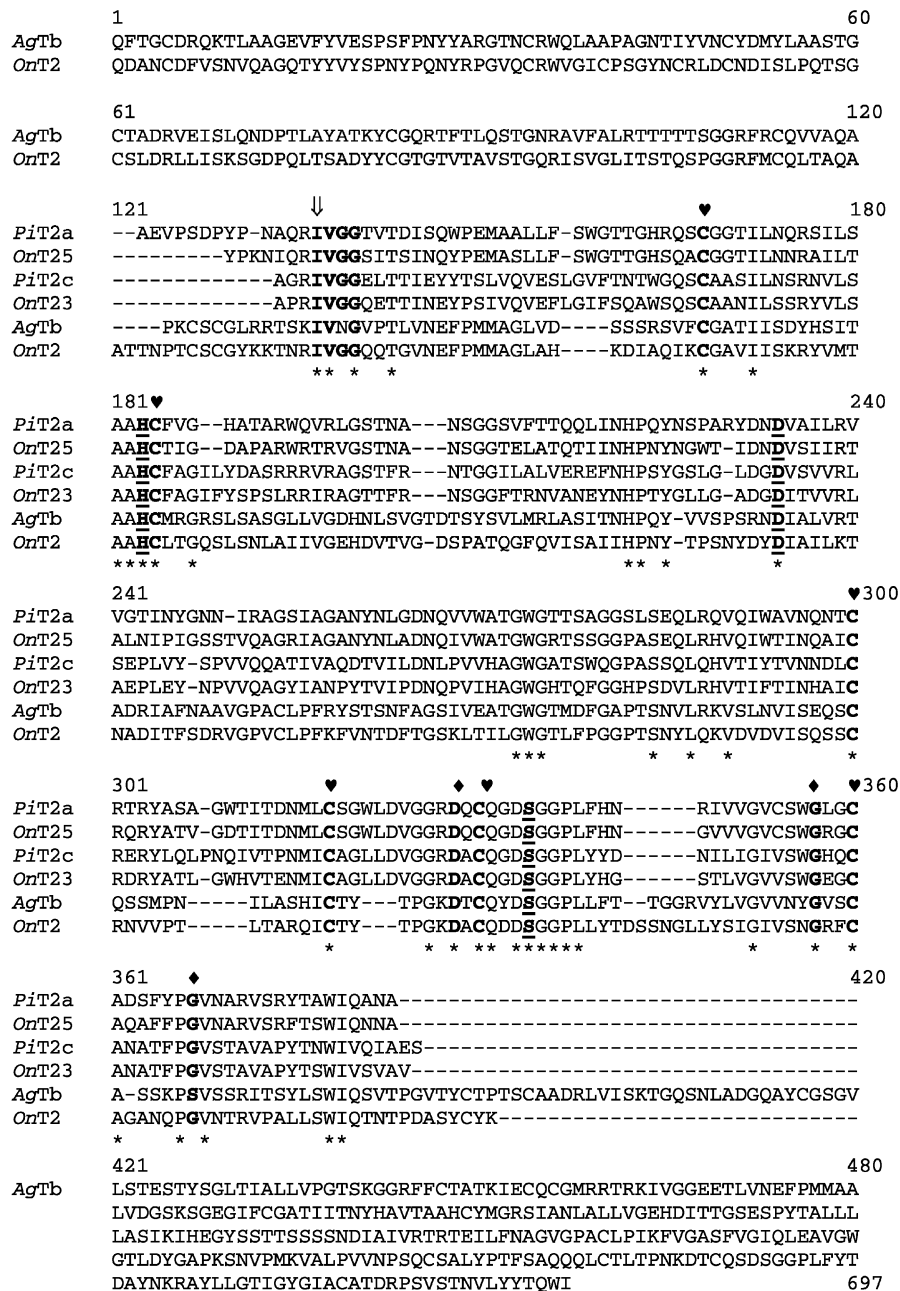


Fig. 4. Alignment of *OnT2*, *OnT23*, and *OnT25* from the Bt-susceptible strain of *O. nubilalis* with three homologous trypsin-like precursor sequences (signal peptide sequences not included). *AgTb* = trypsin-like sequence from *A. gambiae* (African malaria mosquito), GenBank accession number: XP_320615; *PiT2* = trypsin-like sequences from *P. interpunctella* (Indianmeal moth), a: AAC36247; c: AAF24227. Functionally important residues, His¹⁸³, Asp²³⁴, and Ser³³⁴, are underlined and indicated with bold letters. Cysteines corresponding to the sites of predicted disulfide bridges are marked with bold letters and (♥) on the top of the sequences. Conserved residues for the trypsin binding pocket, Asp³²⁸, Gly³⁵⁷, and Gly³⁶⁷, are indicated with bold letters and (♦) on the top. Identical residues among all six sequences are indicated with (*) at the bottom of the sequences. The arrow (↓) represents the *N*-terminal residues of the active trypsin. Hyphens indicate sequence alignment gaps.

cDNAs (*OnT2*, *OnT23*, and *OnT25*) encoding full-length trypsin-like proteinases, within and between the Dipel-resistant and -susceptible *O. nubilalis* strains. Although a number of nucleotide differences were found in resistant and susceptible strains, the differences were also observed for all three cDNAs in individual insects

within each strain. The replacement of proline by histidine at position 172 in the amino acid sequence of *OnT23* may have significant effects on the entire molecular structure and thus could affect its stability and catalytic activity. However, this variation occurred in both strains and did not correlate to resistance. Thus,

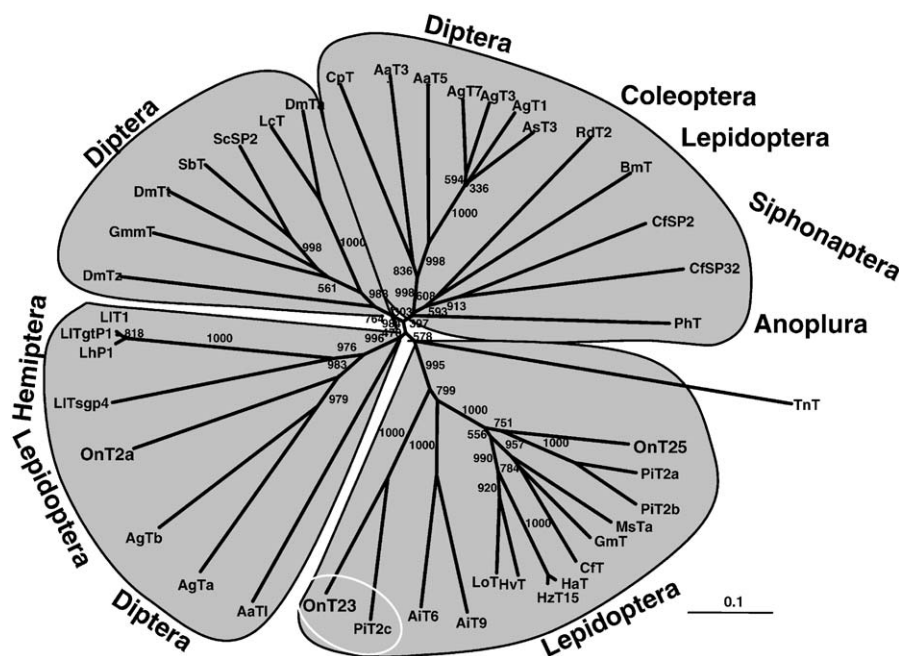


Fig. 5. An unrooted phylogenetic tree of deduced amino acid sequences of insect serine proteinases by the neighbor-joining method. The name consists of the abbreviation of the species (first letter of the genus name followed by the first letter of species name) and proteinase: T for trypsin, P for undefined proteinase and SP for serine proteinase. The bootstrap values with 1000 trials are indicated on branches. Sequences: *AaT*: *Aedes aegypti* (yellow fever mosquito), 1—GenBank accession No. [AAO43403](#), 3—[P29786](#), 5—[P29787](#); *AgT*: *Anopheles gambiae*, 1—[P35035](#), 3—[P35037](#), 7—[P35041](#), a—[XP_320619](#), b—[XP_320615](#); *AtT*: *Agrotis ipsilon* (black cutworm moth), 6—[AAF74732](#), 9—[AAF74733](#); *AsT3*: *Anopheles stephensi* (malaria mosquito), [AAB66878](#); *BmT*: *Bombyx mori* (silkworm), [S32794](#); *CfSP*: *Ctenocephalides felis* (cat flea), 2—[AAD21829](#), 32—[AAD21834](#); *CfT*: *Choristoneura fumiferana* (spruce budworm), [AAA84423](#); *CpT*: *Culex pipiens pallens* (northern house mosquito), [AAK67462](#); *DmT*: *Drosophila melanogaster* (fruit fly), a—[NP_476771](#), t—[P42278](#), z—[NP_523691](#); *GmT*: *Galleria mellonella* (greater wax moth), [AAK81696](#); *GmmT*: *Glossina morsitans morsitans* (tsetse fly), [AAF91346](#); *HaT*: *Helicoverpa armigera* (cotton bollworm), [CAA72956](#); *HvT*: *Heliothis virescens* (tobacco budworm), [AAF43708](#); *LcT*: *Lucilia cuprina* (Australian sheep blowfly), [P35044](#); *LhP1*: *Lygus hesperus* (western tarnished plant bug), [AAK71125](#); *LIT*: *Lygus lineolaris* (tarnished plant bug), 1—[AAL57371](#), sgp4—[AAP12675](#), gtp1—[AAP12676](#); *LoT*: *Lacanobia oleracea* (bright-line brown-eye moth), [CAA07611](#); *MsTa*: *Manduca sexta* (tobacco hornworm), [P35045](#); *OnT*: *Ostrinia nubilalis*, 2a—[AAR98920](#), 23—[AAR98919](#), 25—[AAR98918](#); *PhT*: *Pediculus humanus* (human body louse), [AAQ75386](#); *PiT2*: *Plodia interpunctella*, a—[AAC36247](#), b—[AAF24225](#), c—[AAF24227](#); *RdT2*: *Rhyzopertha dominica* (lesser grain borer), [AAD31268](#); *SbT*: *Sarcophaga bullata* (grey fleshfly), [P51558](#); *ScSP2*: *Stomoxys calcitrans* (stable fly), [AAC39131](#); *TnT*: *Trichoplusia ni* (cabbage looper), [A56593](#).

it appears that these differences are polymorphisms in a heterogonous insect population and are not responsible for lower trypsin-like proteinase activity in the Dipel-resistant *O. nubilalis* strain.

OnT2 contains multiple lysine residues in contrast to most other lepidopteran trypsins (including *OnT23* and *OnT25*) that have only arginine basic amino acid residues. This difference may have resulted from the evolutionary divergence of *OnT2* from *OnT23* and *OnT25*. The bias in favor of arginine and against lysine in lepidopteran trypsins may help to stabilize the protein in the extremely alkaline conditions in the midgut, because arginine has a higher pK_a (12.48) relative to lysine ($pK_a = 10.53$) (Peterson et al., 1994). It is possible that *OnT2* may be expressed in different regions of the gut and has different physiological functions from those of *OnT23* and *OnT25*.

Bernardi et al. (1996) purified a trypsin-like proteinase from *O. nubilalis* larvae with a molecular mass of 24,650 Da as determined by mass spectrometry, similar

to the predicted molecular mass of 24,737 Da for *OnT25*. The first 20 N-terminal amino acid residues of the two mature proteinases (underlined with short bar in Fig. 1C) were identical, except for a serine residue (squared) in *OnT25* and a threonine in the purified proteinase. Thus, it is likely that *OnT25* encodes the purified trypsin by Bernardi et al. (1996) and probably is one of the major digestive trypsins in this insect. In our previous analysis of *O. nubilalis* larval gut proteinases, we identified three $N\alpha$ -benzoyl-L-arginine p -nitroanilide-hydrolyzing activities (Li et al., 2004a). The apparent molecular masses of the three proteinases were estimated to be 34,000, 85,000, and 240,000 Da. The 34,000-Da proteinase was slightly larger than the theoretical molecular masses of mature trypsin-like proteinases of *OnT2*, *OnT23*, and *OnT25* (25–26,000 Da). In the activity blot analyses, however, proteins were resolved on gels in sample and running buffers containing SDS, which can only provide estimates of masses, and the samples were neither heated nor reduced, which may

account for some of the discrepancies in molecular mass. Serine proteinases may resist SDS denaturation (Hames and Rickwood, 1990; Coligan et al., 2000), especially at low temperature (4°C), and may affect migration patterns in the gels. Other explanations include the association of lipids with proteinases, as well as protein oligomerization, resulting in shifts in molecular masses (Houseman and Chin, 1995; Bolognesi et al., 2001).

Our study provides new insights into Bt resistance management strategies. Resistance mediated by reduced activation of Bt protoxin by trypsin-like proteinases may occur in field populations of *O. nubilalis* and other insect species, especially when Bt protoxin-based products, such as Bt spray formulations, are used to control pests. However, studies to date indicate that Bt-resistant *O. nubilalis* strains from various geographical locations are unable to survive on Bt-transgenic corn (Huang et al., 2002; Siqueira et al., 2004). Transgenic Bt corn expresses a truncated version of the CryIAb protein instead of a full-length protoxin (Koziel et al., 1993; Committee on Environmental Impacts Associated with Commercialization of Transgenic Plants, 2002; Andow and Hilbeck, 2004). If the truncated protein is a fully activated toxin, transgenic plants would be protected from this type of proteinase-mediated resistance in insects. This form of resistance could be relatively common in field populations since this strain was one of five selected strains originally exposed to selection pressure and all five strains responded similarly with reduced susceptibility to Dipel (Huang et al., 1997). These findings explain at least in part the lack of resistance to Bt transgenics in field populations.

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